Environmental Health Perspectives
Vol. 80, pp. 149–160, 1989

Multistep Process of Squamous Differentiation in Tracheobronchial Epithelial Cells *In Vitro*: Analogy with Epidermal Differentiation

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The lung, in particular the bronchial epithelium, is a major site for tumor formation in humans. Environmental factors, such as cigarette smoke, in conjunction with genetic factors are important determinants in this disease. Malignant cells exhibit alterations in their control of proliferation and differentiation. It is believed that the acquisition of defects in the regulation of these processes is important in the process of carcinogenesis. A clear insight into the basic mechanisms of the regulation of proliferation and differentiation is required to understand the molecular mechanisms involved in tumor development and in other pathological conditions. Studies using *in vitro* cell culture systems of tracheobronchial epithelial cells provide useful models in which to study the regulation of differentiation and proliferation. The clonogenic cells derived from the treacheobronchial epithelium are pluripotent: They have self-renewal capacity and can differentiate along either a normal, mucosecretory, or a squamous cell pathway. Squamous differentiation in tracheobronchial epithelial cells has many morphological, biochemical, and regulatory properties in common with epidermal differentiation. This pathway of differentiation is a multistep process consisting of at least three stages. In the initial stage, cells become committed to terminal cell division. This is followed by the expression of the squamous differentiated phenotype and finally cornification. Various factors, such as several growth factors, retinoids, calcium ions, and phorbol esters, regulate the program of differentiation at different stages. Studies have indicated that the controls of proliferation and differentiation are interrelated. Cell lines established from tracheobronchial epithelial cells expressing SV40 large T-antigen, as well as carcinoma cell lines, exhibit altered responses to growth and differentiation regulatory factors. Alterations in the commitment to terminal cell division must be a crucial step in the transition of a normal to a malignant cell.

Introduction

The tracheobronchial lining consists of a pseudostratified epithelium (*1,2*), which has been shown ultrastructurally to contain a diverse population of cell types, the major ones being ciliated, basal, and secretory cells (mucous, Clara, and/or serous cells, depending on species). In contrast to ciliated cells, which are considered to be terminally differentiated cells, the basal, Clara, and mucous-secreting cells have proliferative capacity (*3–11*). Next to their secretory function, the Clara and mucous-secreting cells are able to differentiate, probably via independent pathways, into ciliated cells (*6,8,10,12*). The role of the basal cells is not yet fully defined. It has been suggested that basal cells do not serve as precursor cells but solely as support cells for the secretory or ciliated cells. The desmosomal junctions between basal and columnar cells would function as anchors for the columnar cells and would be necessary for their attachment. Recent studies using isolated basal cells from the rabbit trachea have indicated that these cells are capable of regenerating a normal, fully differentiated epithelium when inoculated into denuded tracheas transplanted on the back of nude mice (*11,13*). These findings appear to suggest that the basal cell has the capacity for self-renewal, as well as the ability to give rise to various differentiated cells, and therefore constitutes a stem cell in the tracheobronchial epithelium.

Under a variety of conditions the tracheobronchial epithelium follows a pathway of squamous cell differentiation. Vitamin A deficiency, toxic or mechanical injury induce hyperplasia that is followed by squamous metapla-

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sia (*14–27*). Under these conditions, the pseudostratified epithelium is replaced by a stratified squamous epithelium. This alternate pathway of differentiation has many morphological and biochemical properties in common with epidermal differentiation, although several differences have been noted as well. The squamous pathway of differentiation can also be observed in many squamous cell carcinomas of the lung (*28–30*).

Several *in vitro* culture systems using tracheobronchial epithelial cells from different species have been developed to gain insight into the mechanisms that regulate the proliferation and differentiation of these cells (*31–40*). A clear insight into the basic mechanisms of these processes is required in order to understand the mechanisms involved in tumor development and other pathological conditions. This review is not aimed at giving a detailed overview of the regulation of differentiation in various tracheobronchial epithelial cells from various species but focuses on the regulation of squamous cell differentiation in the rabbit tracheal and human tracheobronchial epithelial cell systems. In addition, since squamous cell differentiation in the tracheobronchial epithelium exhibits many characteristics of epidermal differentiation, in several instances a comparison is made with the differentiation process of human epidermal cells.

Clonogenic Tracheobronchial Epithelial Cells Are Pluripotent

Several *in vitro* culture systems of tracheobronchial epithelial cells have been developed to study the regulation of differentiation of these cells (*31–40*). Our laboratory has been concentrating on the epithelial cells from the rabbit trachea as an *in vitro* model system (*39,41–44*). These cells can be grown in primary culture in a defined medium consisting of Ham's F12 medium supplemented with transferrin, epidermal growth factor (EGF), and insulin. Although insulin is required for proliferation of these cells, insulin growth factor 1 (IGF_1), stimulates growth at a concentration that is 100-fold lower than that of insulin, suggesting that both of these growth factors operate via the IGF_1-receptor.

Rabbit tracheal epithelial cells proliferate rapidly with a doubling time of approximately 19 hr. These cells appear morphologically and biochemically similar to tracheal basal cells (A. M. Jetten, unpublished). Cells exhibit a large nucleus to cytoplasm ratio and contain perinuclear tonofilaments. The keratin profile of these cells is identical to that of cultured cells derived from a 95% pure basal cell preparation and similar to that of basal cells in other tissues such as the epidermis. Isolated, single colonies of these clonogenic rabbit tracheal epithelial cells are able to repopulate denuded tracheas transplanted onto the back of nude mice giving rise to a fully differentiated, normal tracheal epithelium (*13*). *In vitro*, these cells have been shown to be able to differentiate into squamous cells or into mucosecretory cells (*43*). These findings suggest that the clonogenic cells in culture are pluripotent: They have the capacity for self-renewal and are able to differentiate along either a mucociliary or a squamous cell pathway. The morphological features and the keratin profile expressed by these cells suggest that they are basal cells or closely related to basal cells (Fig. 1).

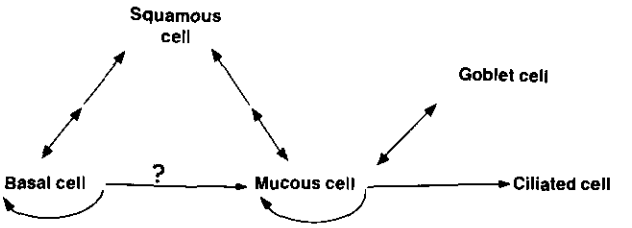

FIGURE 1. Tentative pathway of differentiation of pluripotent tracheobronchial epithelial cells. Clonogenic tracheobronchial epithelial cells have the capacity for self-renewal and appear able to differentiate along either squamous cell or mucosecretory pathways. The mucosecretory cell retains proliferative capacity and can undergo maturation into a Goblet cell or terminally differentiate into a ciliated cell. Morphological characteristics and the expression of keratin proteins suggest that the clonogenic cell may be closely related to the basal cell.

Expression of the Squamous-Differentiated Phenotype

Squamous differentiation in tracheobronchial epithelial cells has many similarities to epidermal differentiation, although several differences have been noted between the two cell systems. The expression of the squamous cell phenotype by tracheobronchial and epidermal epithelial cells in culture is characterized by morphological and biochemical markers. When undergoing squamous differentiation, cells obtain a squamous morphology, contain increased tonofilaments, form increased number of desmosomal junctions, and exhibit a decreased nucleus to cytosol ratio, which is related to increased cell size (*33,45–48*).

At a later stage in this differentiation process, cells undergo cornification (*33,39,46,49–51*), which is characterized by the formation of cross-linked envelopes, consisting of a layer of cross-linked protein, about 100 A thick, deposited just beneath the plasma membrane. Cornification is accompanied by degradation of the cellular organelles. Several factors are involved in the formation of the cross-linked envelope: protein precursors that form the constituents of the envelope and transglutaminase type I (epidermal transglutaminase), the enzyme that catalyzes the covalent linkage between the precursors (*52–56*). Involucrin (*57,58*) and a 190 kD protein (*59*) have been identified and characterized as precursors of the cross-linked envelope in human epidermal keratinocytes. Involucrin has also been found in differentiating human tracheobronchial epithelial cells (*60,61*). The involucrin message has been recently cloned and sequenced (*62*). In rabbit tracheal epithelial cells, a 36 kD protein has been identified as a constituent of the cross-linked envelope (Smits and Jetten, unpublished). In the epidermis, the induction of involucrin and transglutaminase synthesis oc-

curs when cells pass from the spinous to the granular layer (*53,58*). In tracheas of vitamin A-deficient hamsters, the presence of transglutaminase is associated with the squamous and cornifying layers in metaplastic foci (*46*).

Recently, the accumulation of cholesterol sulfate has been shown to be associated with squamous cell differentiation in tracheobronchial and epidermal epithelial cells in culture (*63–65*). The increase in cholesterol sulfate has been related to an increase in the enzyme cholesterol sulfotransferase (*68*). Although a function for the cholesterol sulfate accumulation in the process of squamous differentiation has not been established, it has been suggested that it may play a role in the modulation of lipid metabolism (*67–69*). In the epidermis, cholesterol sulfate accumulation, like the induction of transglutaminase type I activity and involucrin synthesis (*53,58,70,71*), is associated with the transition of the cells from the spinous to the granular layer. *In vitro* as well as *in vivo*, the induction of these biochemical parameters precedes the formation of the cross-linked envelope, indicating that still other factors are involved in envelope formation. It is likely that the synthesis or modulation of other precursors or increased Ca^{2+} concentration, needed for the activation of transglutaminase, might be required (*59,72*).

The expression of keratin intermediate filament proteins is dependent on the cell type and the differentiated state of cells. The regulation of keratin expression in epidermal and tracheobronchial epithelial cells is complex. Basal cells isolated from the rabbit trachea express three major keratins: keratins K5 (58 kD), K14 (50 kD), and K19 (40kD) (*73*). Keratins K5 and K4 are also the major keratins in epidermal basal cells. Epidermal and tracheobronchial epithelial cells in culture express two additional major keratins, keratins K6 (56 kD) and K16 (48 kD) (A. M. Jetten et al., unpublished; *74–76*). The expression of keratins K6 and K16 appears to be associated with the hyperproliferative state of these epithelial cells. Rabbit tracheal epithelial cells in culture exhibit increased expression of the keratins K6, K16, and K13 (46 kD) during squamous cell differentiation (*39*; A. M. Jetten et al., unpublished).

In the skin, epidermal cells undergo keratinization and start to express keratins K1 and K2 (67 and 65 kD) and K10 and K11 (56.5 and 56 kD) (*74,77,78*). However, tracheobronchial epithelial cells undergoing squamous differentiation *in vivo* or *in vitro* appear not to express these keratins (*61,79,80*). Human epidermal keratinocytes in monolayer culture in serum-free medium also do not express keratins K1, K2, K10, and K11 during squamous differentiation, indicating that these cells under this condition do not undergo complete differentiation. However, cells can undergo complete differentiation when grown in the presence of 3T3 feeder cells or on a collagen gel while exposed to air (*77,81*). The induction of transglutaminase and cholesterol sulfate on one hand and the expression of keratins K1, K2, K10, and K11, as well as the synthesis of filaggrin, appear to constitute two different, possibly consecutive, steps in the differentiation process that are regulated differently.

To study the regulation of gene expression during squamous differentiation of tracheobronchial epithelial cells, a cDNA library was constructed from cultured rabbit tracheal epithelial cells that had undergone squamous differentiation (*82*). Screening of the library was aimed at identifying RNAs that are abundant in squamous differentiation cells and not expressed in undifferentiated, proliferative cells. Two different recombinants, designated SQ10 and SQ37, were obtained that hybridize to mRNAs 1.0 and 1.25 kb in length, respectively. These RNAs are present at approximately 50-fold higher levels in squamous cells than in proliferative cells. The identity of these gene products is not known, and work is in progress to sequence the SQ10 and 37 inserts.

Commitment to Terminal Cell Division

Coupling between Growth Control and Regulation of Differentiation

Rabbit tracheal epithelial cells in monolayer culture undergo squamous differentiation when reaching the confluent phase of the growth curve (*43*). Recently, we have proposed a multistep model for this program of differentiation composed of at least three distinct stages: An initial step involving commitment to terminal cell division, a second stage during which differentiated characteristics are expressed, and finally cornification (Fig. 2) (*41–44*). This model not only applies to rabbit tracheal epithelial cells but also to a large extent to monolayer cultures of human tracheobronchial and epidermal cells grown in serum-free media (*33,64,83–85*). The commitment to terminal cell division is characterized by the irreversible reduction in colony-forming efficiency (ability of cells to form colonies when subcultured at low density) and an accumulation of the cells in G_0/G_1 phase of the cell cycle (*43,85*). The reduction in colony forming efficiency has been proposed as a marker for the conversion of the proliferative, undifferentiated cell to one committed to terminal differentiation. Terminal cell division in rabbit tracheal and human bronchial epithelial cells not only occurs at confluence but can be induced in cultures during the exponential growth phase when cells are treated with the phorbol ester phorbol myristate acetate (PMA) (A. M. Jetten, unpublished; *43,86,87*) or when the growth factors EGF and insulin are removed from the medium. Confluency, withdrawal of growth factors, or the addition of phorbol esters can also induce differentiation in epidermal cells (*85,88–92*).

In normal tracheobronchial and epidermal epithelial cells, an inverse relationship exists between proliferation and expression of the squamous differentiated phenotype. A state of irreversible growth arrest not only precedes the expression of the squamous differentiated phenotype but appears to be an obligatory step before expression of this differentiated phenotype can occur. Little is known about the process of terminal cell division or the molecular mechanisms involved in the coupling of the controls of growth and differentiation in these cells. It is likely that specific changes in gene expression accompany the commitment to terminal cell division. Activation of specific

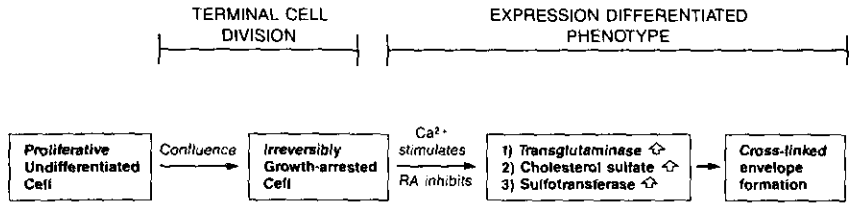

FIGURE 2. Multistep process of squamous cell differentiation in tracheobronchial and epidermal epithelial cells. During the exponential phase of the growth curve cells are hyperproliferative. A confluence cells become irreversibly growth-arrested (terminal cell division) and start to express a squamous differentiated phenotype characterized by squamous morphology, induction of transglutaminase type I activity and accumulation of cholestrol sulfate followed by cross-linked envelope formation. High Ca^{2+} concentration or retinoids (RA) do not effect terminal cell division but influence the expression of the differentiated phenotype.

genes (suppressor or antiproliferative genes) that irreversibly block the proliferation of the cell might be involved, as well as the suppression of genes whose function it is to maintain the proliferative potential of the cells. The mechanisms which underlie the induction of terminal cell division during squamous cell differentiation may be similar to those which operate to induce terminal cell division during senescence. Recently, the existence of abundant antiproliferative mRNAs has been demonstrated in senescent human fibroblasts (*93*). Alterations in the expression of the same or closely related genes may be involved in both of these processes.

Action of Retinoids

As early as 60 years ago (*15*), retinoids were recognized as modulators of differentiation in several epithelial tissues including the tracheobronchial epithelium and the epidermis (*94,95*). Studies with whole animals and *in vivo* organ culture models showed that during vitamin A deficiency the tracheobronchial epithelium undergoes extensive squamous metaplasia (*14–16,20,23,24*). This process is reversible: after treatment with retinoids the normal mucociliary epithelium is restored (*12,23*). In other epithelial tissues as well, retinoids have been shown to inhibit squamous differentiation (*94,95*). Several *in vitro* cell culture systems have been used to study the action of retinoids on squamous differentiation (*43,96–98*). In the multistep program of squamous cell differentiation proposed in Figure 2 retinoids affect very specific stages of squamous differentiation whereas other stages are insensitive to retinoids. Retinoids do not block the commitment to terminal cell division in tracheobronchial or epidermal cells induced by confluence, withdrawal of EGF and insulin or addition of TGF-β or PMA (*43,64,73*); however, retinoids do block the expression of the differentiated phenotype. Retinoids inhibit the induction of transglutaminase type I (*54,99–102*) and cholesterol sulfotransferase activities, inhibit the accumulation of cholesterol sulfate (*63–66*), and inhibit cornification in several *in vitro* tracheobronchial and epidermal cell systems (*39,43,103–105*). In addition, retinoids markedly alter keratin expression in these cells. In epidermal keratinocytes, retinoids inhibit the expression of the 67–65 kD (K1 and K2) and 56, 56.5 (K10 and K11) kD keratins, which are markers for keratinization (*77,106*).

In tracheobronchial and epidermal cells, retinoids inhibit the synthesis of keratins K5 (58 kD), K6 (56 kD), and K16 (48 kD) and increase the synthesis of keratin K19 (40 kD) (*75,80,107*). In rabbit tracheal epithelial cells, retinoic acid also inhibits the increase in two squamous cell specific mRNAs, SQ10 and SQ37 (*82*). The differential effect of retinoids on the commitment to terminal cell division and expression of the differentiated phenotype suggest that these two processes are controlled separately. However, as stated earlier, regulation of these two processes appears to be linked.

At what level retinoids control the expression of the differentiated phenotype remains to be established. The effects of retinoids on keratin expression is related to alterations in the levels of corresponding mRNAs (*106,107*). Also the inhibition of the expression of SQ10 and SQ37 is related to changes in their mRNA levels (*82*). Retinoids may act at many levels, affecting gene expression at the transcriptional level as well as affecting the transport or stability of certain mRNAs. Chytil and Ong (*108*) have proposed that retinoids act like steroid hormones via specific receptors that interact with specific sequences in the chromatin, thereby altering gene transcription. Chytil and Ong (*108,1091*) and later many other investigations (*95,110,111*) have identified specific cytosolic binding proteins for retinoic acid and retinol, designated CRABP and CRBP, respectively, in many tissues and cell lines. For embryonal carcinoma cells, mutant cell lines have been isolated that do not respond to retinoic acid and lack CRABP, suggesting a role for CRABP in the mechanism of action of retinoids (*112,113*).

However, in many cell lines that do respond to retinoids, such as in promyelocytic leukemia HL60 cells (*111,114*), no detectable levels of CRABP have been found. In addition, certain benzoic acid derivatives of retinoic acid (Chseries) which are equally or more active than retinoic acid, are unable to bind to CRABP (*100*). These studies indicate that CRABP may not be essential for the action of retinoids and that other receptors may be involved in the mechanism of action. Recently, a human retinoic acid receptor (RAR) has been identified that belongs to a family of nuclear receptors (*115,116*). This receptor protein is homologous to the receptors for steroid hormones, thyroid hormones, and vitamin D3 and appears to be a retinoic acid-inducible, *trans*-acting enhancer factor. This receptor has a high affinity for retinoic acid and at least a

100-fold lower affinity for retinol (*115*) and is present in low abundance (several thousand molecules/cell). It is not known whether a similar nuclear receptor exists for retinol. It is possible that the action of the Ch-series of retinoids is explained by the binding of these analogs to the RAR protein.

Whether CRABP and RAR cooperate in the mechanism of action of retinoids remains to be investigated. In Figure 3, two possible mechanisms of action are illustrated that might be involved in the regulation of differentiation by retinoids. Figure 3*A* shows a model in which CRABP translocates retinoic acid to the nucleus and then transfers the retinoic acid to the nuclear receptor. Such a model would explain the absence of responsiveness in embryonal carcinoma cells lacking CRABP activity (*112,113*) if CRABP is essential for retinoic acid action. The latter, however, would not be in agreement with findings showing that in certain cell systems, cells respond to retinoic acid in the absence of CRABP (*114,115*). The activity of the retinoic acid analogs of the Ch-series, which do not bind to CRABP, also indicate that CRABP is not essential for the action of retinoids. It is possible that the analogs of the Ch-series bypass CRABP and bind directly to RAR. In the second model (Fig. 3*B*), CRABP is not involved in the mechanism of action and retinoic acid binds to RAR directly. The cellular localization of RAR is not known; possibly all the RAR is associated with the nucleus. Alternatively, RAR is localized in the cytosol and after binding retinoic acid is translocated to the nucleus. This model would not explain why in mutant embryonal carcinoma cell lines that lack CRABP, cells do not respond to retinoic acid. Future studies will be needed to determine what role CRABP plays in the mechanism of action of retinoids.

During differentiation, cells undergo a complex program of changes in gene expression. Interaction of the RAR-RA complex with specific enhancer sequences could activate or repress the transcription of specific genes associated with the differentiated phenotype in tracheobronchial and epidermal cells (Fig. 4). Some of these genes might represent transcriptional factors which then regulate the transcription of other genes whose expression is associated with the differentiated phenotype such as genes for keratins, transglutaminase type I, and sulfotransferase. Changes in the activity of specific transcriptional factors have been demonstrated during retinoic acid-induced differentiation of embryonal carcinoma cells (*117-118*). The transcription of such factors might be under direct control by RA-RAR and determine the expression of many other genes during the differentiation process (Fig. 4). It seems certain that the activity of specific transcriptional factors will be shown to play a key role in regulating the complex changes in gene expression that occur during differentiation.

Role of Calcium

Calcium ions can influence various aspects of squamous cell differentiation in tracheobronchial epithelial and epidermal cells. In murine epidermal keratinocytes, a calcium concentration of $> 100\ \mu M$ in the medium induces terminal differentiation (*119-121*). In contrast, tracheobronchial epithelial and human epidermal cells exhibit optimal growth at calcium concentrations $> 100\ \mu M$ (*33,43,84,85*) and maintain a high colony-forming efficiency. However, high calcium does induce a reversible morphological change in these cells (*33,43,84,85*): cells es-

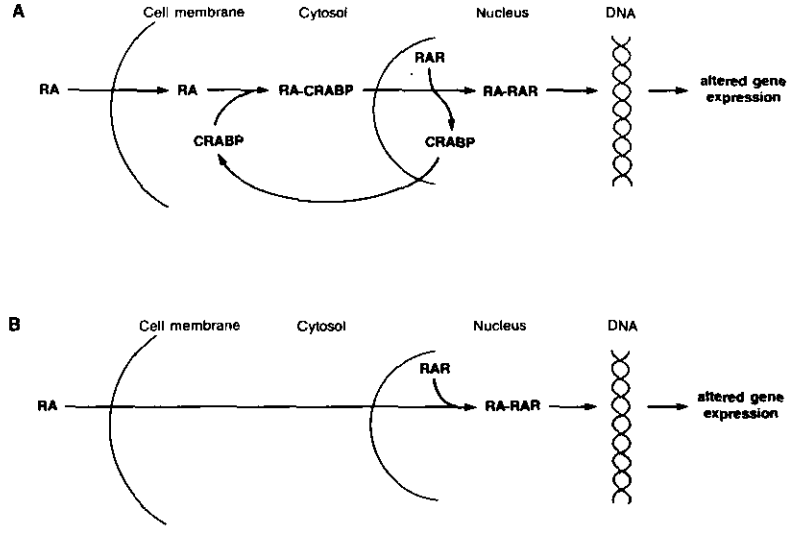

FIGURE 3. Mechanisms of action of retinoic acid. (*A*) Cooperative model. Retinoic acid (RA) binds to the cytosolic retinoic acid binding protein (CRABP) and is translocated to the nucleus where retinoic acid is transferred to the nuclear retinoic acid receptor (RAR). This complex interacts with specific enhancer sequences modulating the transcriptional activity of certain genes. (*B*) Noncooperative model. The CRABP is not involved in the mechanism of action of retinoic acid. Retinoic acid interacts directly with RAR in the cytosol or nucleus. The RA-RAR complex interacts subsequently with chromatin thereby altering gene expression.

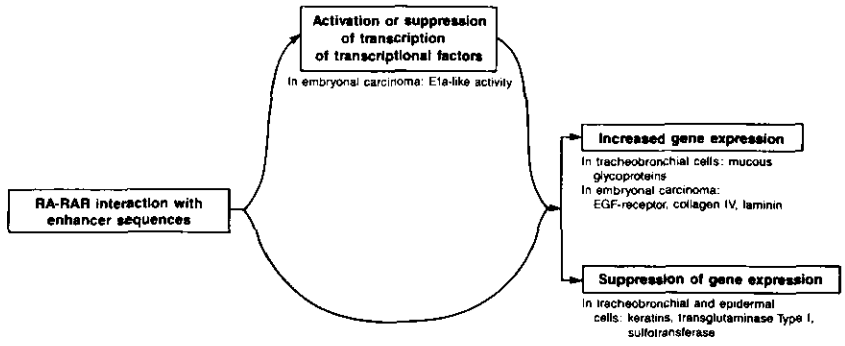

FIGURE 4. Direct and indirect modulation of differentiation markers by retinoic acid. Regulation of gene expression by retinoic acid can occur in a direct or indirect manner. Retinoic acid-nuclear retinoic acid receptor (RA-RAR) complex interaction with specific enhancer sequences could activate or repress the transcription of specific genes (direct control) associated with the differentiated phenotype such as genes for mucin, keratins, transglutaminase type I and sulfotransferase. Some of these genes might represent transcriptional factors which then regulate the expression of other genes (indirect control).

tablish cell-cell contacts and desmosomal junctions (*119*). High calcium alone does not induce terminal cell division in proliferating tracheobronchial or human epidermal cells; however, when cells undergo terminal cell division at confluence, high calcium concentrations stimulate the expression of the squamous differentiated phenotype (*43,63,64,96,102*). High calcium concentrations increase transglutaminase type I activity and the accumulation of cholesterol sulfate and increase the rate of cross-linked envelope formation in both tracheobronchial and epidermal cells. The mechanism by which calcium modulates differentiation is still poorly understood.

Transforming Growth Factor β

Transforming growth factor β (TGF-β) is a polypeptide hormone that is expressed and released by many normal and neoplastic cells (*123,124*). It consists of two 12 kD chains linked by disulfide bonds and is synthesized as part of a larger secretory precursor (*123*). The total amino acid sequences of human and mouse TGF-β are known from their complementary cDNA and show a remarkable homology (*125*). TGF-β is a multifunctional peptide that controls cell proliferation, differentiation and other functions in many cells types (*126–130*). The cellular responses to TGF-β are mediated by specific cell-surface receptors (*131,132*); however, little is known about the signal transduction mechanism of the action of TGF-β.

The effect of TGF-β on tracheobronchial epithelial cells appears to depend on the cell density of the cultures. At low density TGF-β induces terminal cell division, and in the presence of high calcium in the medium cells start to express a squamous differentiated phenotype (*133,134*). At high densities cells become much more resistant to TGF-β; whether this is due to cell-cell interaction or the release of an endogeneous factor into the medium is not known. TGF-β is synthesized by tracheobronchial epithelial cells as its precursor. This factor might play an important role in maintaining the balance between proliferation and differentiation in the tracheobronchial epithelium.

Phorbol Esters

Phorbol esters elicit a diverse set of responses in tracheobronchial and epidermal cells. In epidermal cells in culture, as well as *in vivo*, the different responses are related to different subpopulations of undifferentiated cells (basal cells) (*135–138*). In one subpopulation (population A) (Fig. 5) phorbol esters induce a proliferative response as indicated by a stimulation in ^{3}H-thymidine incorporation and induction of ornithine decarboxylase activity. In the other subpopulation (population B) (Fig. 5) phorbol esters induce terminal cell division and expression of the differentiated phenotype as indicated by the induction of transglutaminase type I activity, accumulation of cholesterol sulfate, and increased cross-linked envelope formation (*88–91,99*).

Population A appears to consist of the smallest cells that have the lowest probability to undergo differentiation. Cells from population A are believed to give rise to population B cells, a process that is probably reversible (*139*). Cultures of human foreskin keratinocytes maintained in serum-free medium and in the absence of feeder layers appear to consist of mostly population B cells, as treatment with phorbol esters does not induced ornithine decarboxylase activity but does induce terminal differentiation in most of the cells (*89–91*). However, when these cells are cultured on feeder layers, a subpopulation of type A cells is maintained (*140–142*). Tracheobronchial epithelial cells in monolayer culture appear largely to behave like population B cells (*64,86,87,143*). After treatment with phorbol esters, cells undergo terminal cell division and start to express type I transglutaminase activity, accumulate cholesterol sulfate, and form cross-linked envelopes (A. M. Jetten, unpublished; *64*). Whether tracheobronchial epithelial cells at one point after isolation contain population A cells is not known.

Cultures of human bronchial epithelial cells have been shown to respond heterogeneously to the phorbol ester PMA (*143*). In freshly isolated rat tracheal epithelial cells, PMA increases the colony forming efficiency, whereas in primary cultures PMA induces terminal differentiation in all cells (*144*). These studies indicate a diversity in the re-

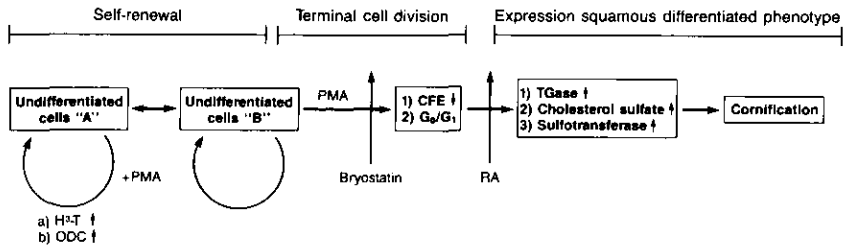

FIGURE 5. Schematic view of the mechanisms by which the phorbol ester PMA, bryostatin, and retinoic acid modulate squamous cell differentiation in tracheobronchial and epidermal epithelial cells. In cell subpopulation A, PMA induces cell proliferation as indicated by increased ornithine decarboxylase activity (ODC) and ^{3}H-thymidine incorporation. Subpopulation A cells can convert reversibly into subpopulation B cells, which respond to PMA by undergoing terminal differentiation. Bryostatin mimics the effect of PMA in subpopulation A cells. In subpopulation B cells, bryostatin antagonizes the induction of squamous cell differentiation by PMA. Bryostatin blocks both the induction of terminal cell division as well as the expression of the differentiated phenotype. Retinoic acid does not influence the commitment to terminal cell division but does inhibit the expression of the differentiated phenotype.

sponse of tracheobronchial epithelial cells to PMA that perhaps is related to different subpopulations of undifferentiated cells similar to population A and B cells. It is not known what molecular changes dictate the different responses of population A and B cells to phorbol esters.

The major action of phorbol esters in cells is mediated by the activation of protein kinase C, which is encoded by a family of closely related genes (*145*). Diacylglycerol, the endogeneous activator of protein kinase C, produces many of the same effects reported for phorbol esters (*146*). Bryostatins, macrocyclic lactones isolated from marine bryozoan *Bugula neritina* (*174*), bind to and activate protein kinase C (*148–152*). Like phorbol esters, they can induce ornithine decarboxylase activity and ^{3}H-thymidine incorporation in subpopulation A mouse epidermal cells (*152*). However, bryostatin does not induce terminal differentiation in subpopulation B mouse epidermal cells, as phorbol esters do (*152*).

In contrast, bryostatin inhibits terminal differentiation induced by the phorbol ester PMA (*152*). In both human epidermal keratinocytes and tracheobronchial epithelial cells, bryostatin also inhibits squamous cell differentiation induced by PMA (A. M. Jetten, unpublished). Bryostatin inhibits both the commitment to terminal cell division induced by PMA as well as the expression of the differentiated phenotype (A. M. Jetten, unpublished). The mechanism of this antagonism is poorly understood. Epidermal and tracheobronchial epithelial cells could express several protein kinase C isoenzymes that could be affected by phorbol esters and bryostatins in different ways in terms of affinity, downregulation, or substrate specificity (*149–152*). Alternatively, bryostatin could act via more than one mechanism: one action is mediated via the activation of protein kinase C that is antagonized by the activation of a yet unknown pathway unrelated to protein kinase C.

Retinoic acid has a quite different effect on the process of terminal differentiation induced by phorbol esters than bryostatins. Although retinoic acid does not inhibit the commitment to terminal cell division induced by phorbol esters in either epidermal or tracheobronchial epithelial cells (A. M. Jetten, unpublished), it does inhibit the induction by PMA of transglutaminase type I activity, the accumulation of cholesterol sulfate and cross-linked envelope (*99*; A. M. Jetten, unpublished). These findings suggest that retinoic acid inhibits expression of the squamous differentiated phenotype. Studies in mouse epidermal cells have shown that retinoic acid can also inhibit the induction of ornithine decarboxylase activity by PMA in population A cells (*153*). These results indicate that retinoic acid can act at more than one stage in the differentiation process.

Figure 5 shows a schematic review of the action of phorbol esters, bryostatin, and retinoic acid on the process of squamous cell differentiation in epidermal and tracheobronchial epithelial cells. Phorbol esters induce terminal cell division in population B cells followed by expression of the differentiated phenotype. Bryostatin blocks the first stage in this differentiation process whereas retinoic acid blocks the second stage.

Hyperplasia, Squamous Metaplasia and Squamous Cell Carcinomas

In the normal pseudostratified tracheobronchial epithelium, a strict balance exists between cellular proliferation and differentiation. This balance is likely maintained via an equilibrium between positive and negative growth regulatory factors and factors controlling differentiation. Studies *in vitro* have shown that proliferation of tracheobronchial cells is dependent on EGF/TGF-α and insulinlike growth factors and is regulated in a negative manner by TGF-β (*34,39,133,134*). Other factors, such as interferons and interleukins, may also regulate proliferation in these cells. It is likely that these positive and negative regulatory factors also play a role in maintaining a balance between proliferation and differentiation *in vivo*.

Cells in the normal tracheobronchial epithelium have a low mitotic rate, as has been established for the basal cells in the skin. Many cells are withdrawn from the cell cycle and remain in the resting (G_0) phase. During vitamin A deficiency or after mechanical or toxic injury, the balance between proliferation and differentiation is disturbed; the epithelium undergoes hyperplasia followed by squamous

metaplasia (*14–27*).

In the case of mechanical and toxic injury this process may be part of a wound healing or tissue repair mechanism. The mechanism underlying the hyperplastic response has not been completely elucidated. Hyperplasia may involve recruitment of the cells in the G_0-compartment and re-entry of these cells into the cell cycle. Altered synthesis or activation of specific positive or negative growth regulators may induce the G_0 to G_1 transition in resting cells and initiate their proliferation. Studies carried out by Stahlman et al. (*154,155*) are in agreement with this concept. Increased levels of EGF, administered IV to fetal lambs, induces hyperplasia and squamous metaplasia in the tracheobronchial epithelium.

The fact that the phase of hyperproliferation (hyperplasia) is followed by a phase in which cells stop proliferating and undergo squamous differentiation (squamous metaplasia) indicates that cellular proliferation is not unlimited but strictly controlled. However, the molecular mechanisms that trigger terminal differentiation *in vivo* have not yet been established. Increased synthesis or activation of TGF-β, which can induce terminal differentiation of tracheobronchial cells *in vitro* (*133,134*), may also be involved in the induction of squamous differentiation *in vivo*.

The lung, in particular the bronchial epithelium, is a major site for tumor formation in humans. Environmental factors, such as cigarette smoke, in conjunction with genetic (hereditary) factors are important determinants in this disease. It is believed that the acquisition of defects in the control of proliferation and differentiation are important steps in the multistage process of carcinogenesis. Alterations in the mechanisms that control hyperplasia and squamous metaplasia are thought to be involved in the development of squamous cell carcinomas. Activation of specific protooncogenes, constitutive synthesis of polypeptide growth factors, decreased responsiveness to negative growth regulators, as well as the loss of antiproliferative or tumor suppressor genes are believed to be the molecular mechanisms involved in the conversion of a normal cell into a transformed cell. For example, tracheobronchial epithelial cells expressing the SV40-large T-antigen have an indefinite life span and are resistant to certain inducers of differentiation (A. M. Jetten, unpublished); activated oncogenes from the *ras*, *myc*, and *raf* families have been found associated with lung carcinomas, and lung carcinoma cell lines have been found to be much more resistant to the growth-inhibitory effects of TGF-β than normal tracheobronchial epithelial cells (*133,134*). Such alterations in gene activity reduce the ability of a cell to become quiescent and arrested in the G_0, reduce its probability to undergo terminal differentiation, and promote uncontrolled proliferation.

Summary

It is clear that the use of *in vitro* cell systems has been very useful in identifying not only various factors that control proliferation and differentiation in tracheobronchial epithelial cells but also several biochemical and molecular markers that are associated with the expression of the differentiated phenotype. Studies have indicated that the program of squamous differentiation is a multistep process. Elucidation of the mechanism of action of these regulatory factors and the mechanism of gene regulation are required to understand the process by which differentiation is controlled. Introduction of transforming genes in normal tracheobronchial cells, yielding cells nonresponsive to differentiation inducers, appear to be a promising approach that may provide insight in the process of carcinogenesis.

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